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The major goals of this study have been to document the usefulness of volumetric MRI as a means of following the growth of plexiform neurofibromas, measuring the rate of growth of plexiform neurofibromas over time, and establishing a consortium of clinical centers to carry out clinical trials of treatment for plexiform neurofibromas in the future. Establishment of this consortium has been considerably more difficult than anticipated, due to the need to have every center approved both by a local IRB and the army IRB. Many minor and a few major differences in wording of informed consents have impeded many centers from completing the IRB approval process. We have tried to streamline this process, now, and expect that 19 centers will be approved by the first quarter of 2001. With this, however, we will have accomplished one of our major goals, to create a consortium of clinical centers, supported by a tissue repository, database, pathology review, MRI review, and statistical support. We have also taken a major step towards demonstration that the volumetric approach can be accomplished in spite of the complexities of imaging of plexiform neurofibromas by showing that three independent observers obtain similar volume measurements. The final goal, that of determining rate of neurofibroma growth, will take longer than expected due to the slow acquisition of IRB approval for all clinical centers, but this is expected to be accomplished as well, albeit after a longer period of study than initially anticipated. The first clinical trial of a farnesyl protein transferase inhibitor is about to begin under the auspices of the National Cancer Institute. We are involved in this study, using the same MRI protocol and tissue repository for that study. It is expected that additional clinical trials will begin over the next several years, and that the approaches and resources of this consortium will facilitate the efforts to test these drugs and determine treatment endpoints in NF1.

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Introduction

This report marks completion of the second year of this project. This year saw a major change in the administrative structure of the project, as the principal investigator moved from Children's Hospital to Brigham and Women's Hospital. This necessitated rewriting the various subcontracts and rebudgeting the contract. Another important change was moving the Pathology Review Facility from Mt. Sinai Hospital in New York to Washington University in St. Louis. This consolidated the tissue banking and pathology review in a single facility. We have also strengthened communications in the network, creating a web site and searchable online databases of project information. The major impediment we have encountered remains the very cumbersome need to have each participating center receive approval from both their local institutional review board and also the Army IRB. A meeting was held recently with the US Army Medical Research and Materiel Command at Ft. Detrick, which should result in a better understanding of the process and more efficient center approval.

Progress Report for Statement of Work by Task

Task 1. Complete development of study infrastructure – Months 1-6

a. IRB approval at all clinical sites

Table 1 lists the participating clinical centers, the principal investigator at each site, and the IRB approval status. The IRB column refers to approval by the local IRB; the "Army" column refers to approval by the army IRB. Several centers have been dropped from the study. These are: Oxford University, University of Leuven, and University of Sao Paulo. Reasons for drop out were lack of follow-through by the local investigator to complete the necessary paperwork for IRB approval. Several new centers have been added, both to replace dropout and to accommodate investigators who have expressed a strong desire to participate. These are: National Cancer Institute, Mayo Clinic, University of Michigan, Toronto Hospital for Sick Children, University of Colorado, University of Manchester, University of Padova, University of Pittsburgh, and University of Texas. We have prepared a package for each new participating clinical center to streamline the process of IRB approval that we hope will expedite this process.

| Center | PI | IRB | Army | Comments |
|---------------------------------------|----------------------|-------------------------------------|-------------------------------------|-------------------------------|
| Children's Hospital Boston - 107 | Bruce Korf | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Children's Hospital Medical Ctr - 173 | Robert Hopkin | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Children's Hospital of Oklahoma - 178 | John Mulvihill | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Children's Memorial Hospital - 177 | Joel Charrow | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Children's National Medical Ctr- 170 | Roger Packer | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Guy's Hospital - 187 | Rosalie Ferner | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Klinikum Nord Ochsenzoll - 160 | Victor-Felix Mautner | <input type="checkbox"/> | <input type="checkbox"/> | send to CD for review |
| Mass General | Mia MacCollin | <input checked="" type="checkbox"/> | <input type="checkbox"/> | wtg for Partners approval |
| Mayo Clinic | Dusica Babovic | <input type="checkbox"/> | <input type="checkbox"/> | submitted protocol & consents |
| Mt. Sinai School of Medicine | Allan Rubenstein | <input type="checkbox"/> | <input type="checkbox"/> | probably not participating |
| National Cancer Institute | Brigitte Widemann | <input checked="" type="checkbox"/> | <input type="checkbox"/> | sent CF to Army 10/20 |
| New Children's Hospital | Kathryn North | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Texas Children's Hospital - 172 | Sharon Plon | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Toronto Hospital for Sick Children | Patricia Parkin | <input type="checkbox"/> | <input type="checkbox"/> | wtg to hear from IRB |
| University of British Columbia - 100 | Jan Friedman | <input checked="" type="checkbox"/> | <input type="checkbox"/> | wtg for protocol amendment |
| University of Colorado | Eva Sujansky | <input type="checkbox"/> | <input type="checkbox"/> | submitted protocol & consents |
| University of Manchester | Gareth Evans | <input checked="" type="checkbox"/> | <input type="checkbox"/> | new PI, EM 12/12 |
| University of Michigan | John Fink | <input type="checkbox"/> | <input type="checkbox"/> | submitted protocol & consents |
| University of Padova | Romano Tenconi | <input type="checkbox"/> | <input type="checkbox"/> | intends to participate |
| University of Pittsburgh | Vinodh Narayanan | <input type="checkbox"/> | <input type="checkbox"/> | submitted protocol & consents |
| University of Texas | Moore & Slopis | <input type="checkbox"/> | <input type="checkbox"/> | sent CF & protocol 8/18 |
| University of Utah - 117 | David Viskochil | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |
| Washington University - 169 | David Gutmann | <input checked="" type="checkbox"/> | <input checked="" type="checkbox"/> | - |

Table 1. Status of IRB approval of participating clinical centers.

b. Complete clinical data entry forms and test electronic transfer of clinical data

Data entry forms were completed by the end of the first year, and have not changed.

c. Organize package of materials for pathology review and tissue repository

This year the pathology review, facility and tissue repository were consolidated into a single site at Washington University. Dr. David Gutmann remains the PI for the tissue repository, and Dr. Arie Perry is the PI for the pathology review. A detailed protocol for submission of tissue specimens has been produced and is available for download on our website. The protocol is provided in Appendix A.

d. Set up listserve and website

The study website is now operational at www.nfstudies.org (figure 1). Major features include general information about the study, a list of participating clinical centers, enrollment and exclusion criteria, and information for centers. In addition there is an MRI database, which is described below.

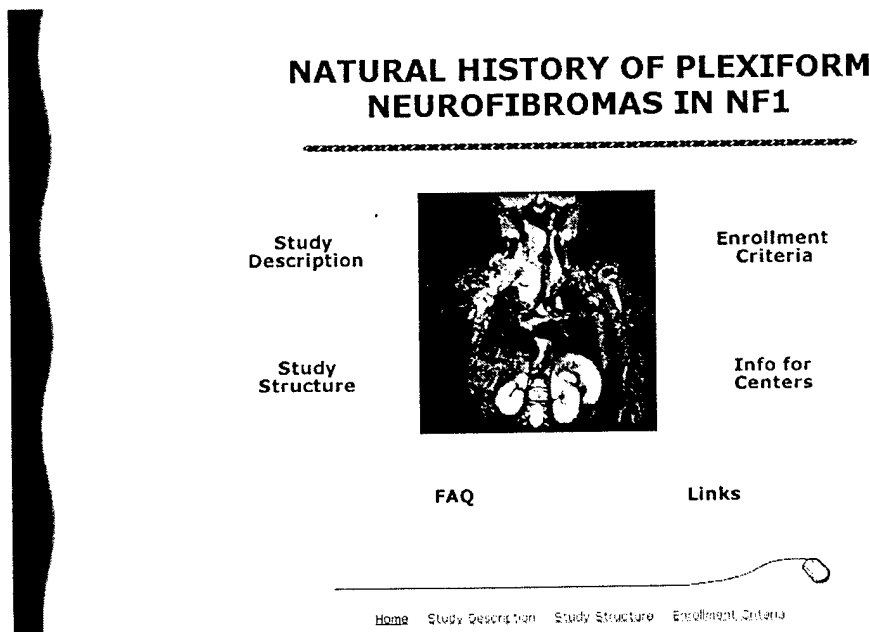


Figure 1. Screenshot of project homepage.

The MRI Database website (figure 2) is a dynamic tool with several useful features. The site was developed for study administrative staff and patient collection centers to perform quality assurance checks on data received by WorldCare. This site is divided into four sections: search for MRI, administrative QA, frequently asked questions, and contact us. The "search for MRI" section allows patient collection centers to view the status of patient MRIs collectively or individually. This form searches all records in the WorldCare MRI Database and returns real-time information pertaining to the scan in question. The center will know when the scan was imported and accepted by WorldCare, the date it was analyzed by a radiologist, its current status, and any comments related to the scan. The "administrative QA" section is intended for study administrative staff to perform random quality assurance checks. This section is password protected to avoid any misuse of information. The "frequently asked questions" section lists common questions regarding image transfer, proper documentation procedures, etc. The "contact us" section provides ways to contact the parties directly involved with maintaining the information on the site.

Search

[Home](#) [Search](#) [FAQ](#) [Contact](#)

Search by Center ID #
(e.g. 114)

Submit

Enter your center ID # display all scans from your center

Search by Patient ID #
(e.g. 114-8998-003)

Submit

Enter a patient ID # and display all MRIs for that patient

If you have questions or comments regarding the website, please click [here](#) to email

Figure 2. Screenshot of search page for MRI database.

e. Test MRI data transfer

The following 15 MRI centers have submitted test data for the NF1 Study either by optical disk or through File Transfer Protocol (FTP):

Children's Hospital
Boston, MA

Center for Human Genetics
Leuven, Belgium

Children's Hospital Medical Center
Cincinnati, OH

Children's Memorial Hospital
Chicago, IL

Children's Hospital of Oklahoma
Oklahoma City, OK

Children's National Medical Center
Washington, D.C.

Guys Hospital
London, UK

Klinikum Nord Ochsensol
Hamburg, Germany

Massachusetts General Hospital
Charlestown, MA

Mount Sinai Medical Center
New York City, NY

Royal Alexandria Hospital
Parramatta, NSW,
Australia

Texas Children's Hospital
Houston, TX

University of British Columbia
Vancouver, British Columbia

University of Utah
Salt Lake City, UT

Washington University
St. Louis, MO

f. Purchase workstation and prepare data entry forms at WorldCare.

The workstation was purchased in November of 1998. Documentation was provided in last year's progress report.

WorldCare has maintained the NF1 Natural History Study infrastructure by ensuring that on site project systems are constantly prepared for data collection and analysis. To this end efforts have been made to bring up to date existing hardware and software responsible for all aspects of project functionality. Included are equipment for sending and receiving images such as optical drives, translators and servers related to file transfer protocol. Additionally, the image analysis suite has been updated and is running on a recently purchased, cutting edge computer. While the image software is 510K approved, WorldCare contracted an independent validation company to guarantee that all analytical and statistical systems are running efficiently. The patient-tracking database has been maintained with small reorganizations designed to more accurately audit patient visit information and data. Previously instituted filing systems, logbooks and binders have been kept current to track both the history and progress of efforts made by all parties in contact with WorldCare.

To accurately reflect the procedural changes made in the NF1 project, WorldCare document control updates and revises standard operating procedure manuals as necessary. These manuals outline procedures for the collection, receiving and analysis of data specific to the study within Good Clinical Practices (GCP) guidelines. The NF1 Collection Center Study Manual and has been distributed to the clinical coordinators at each MRI facility in the study.

g. Prepare project monitoring flow sheet at Brigham and Women's Hospital

The overall progress of the project is monitored on a spreadsheet kept in the PI's offices at Partners HealthCare System (Brigham and Women's Hospital).

h. Prepare recruitment letters for study subjects

This was addressed last year.

i. Publicize study to NF community

The study continues to be publicized in newsletters of the National Neurofibromatosis Foundation and of NF, Inc.

Task 2. Recruitment of Study Subjects – Months 6-12

- a. Centers contact prospective study subjects**
- b. Enrollment of study subjects**
- c. First MRI and clinical data received**

Tasks a-c continue to be impeded by the delays in obtaining IRB approval for participation of the study centers. The need to obtain approval for each site from two IRB's creates an exceedingly cumbersome mechanism that results in delays of many months in the approval of each center. Because of this, some centers have required more than one year to be approved, some still are not approved, and others have dropped out. Currently, 10 centers have been fully approved, 5 have local IRB approval and await final army approval, and 9 more have been added more recently. This represents substantial progress compared with the end of year 1, when only 4 centers were approved. The rate of subject accrual is displayed in figure 3. In general there has been a surge of recruitment following approval of a new center. For example, the increase between Q6 and Q7 was attributable to the successful negotiation of the agreement between the Army IRB and the University of Utah, which required more than one year to complete.

Progress in recruitment to the six study categories is shown in Table 2. The goal is to have 50 subjects recruited into each category. Two categories are nearly closed. We have now streamlined the process of IRB approval, by having a boilerplate informed consent that anticipates most of the common problems that have been encountered by centers entering the study to date. We anticipate that this will expedite the approval of the nine pending centers. This will nearly double the number of participating centers, and, we believe, will allow us to recruit the full number of subjects initially anticipated (300). It is our goal to have this done by the summer of 2001. This will necessitate extension of the period of MRI accrual and analysis. Since

accrual has been slower than anticipated, there are substantial unexpended funds that will make this possible.

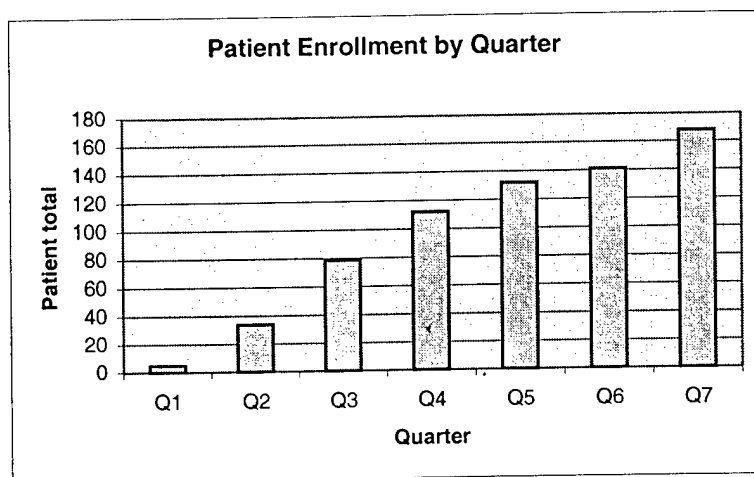


Figure 3. Cumulative subject enrollment by quarter from start of study to present.

| Study Category | | Number Recruited |
|---|----------------|------------------|
| Head & Neck | < 18 years old | 48 |
| | > 18 years old | 16 |
| Trunk & Extremity Externally Visible | < 18 years old | 44 |
| | > 18 years old | 35 |
| Trunk & Extremity Not Externally Visible | < 18 years old | 12 |
| | > 18 years old | 8 |
| Total | | 163 |

Table 2. Number of subjects recruited by study category.

d. Review of clinical entry criteria

Entry and exclusion criteria were reviewed in a meeting held in February 1999 at the Banbury Center in Cold Spring Harbor, N.Y. A follow-up meeting of the steering committee and participating clinical centers was held in Aspen, CO in June, 2000. No changes were made in the entry criteria at that meeting.

e. Test of inter-observer reproducibility of designation of tumor margins by MRI

Twelve MRI scans were chosen for the reproducibility study, including scans from each of the study sites (head and neck, trunk and extremities). Moreover, the tumors were selected so that in half the margins could be easily identified ("discrete") and in half the margins were more diffuse (figure 4). The goal of the study was to determine the consistency with which tumor margins are drawn by three independent observers, and how this influences estimations of tumor volume. The three observers included the two study radiologists, Drs. Tina Young Poussaint and Diego Jaramillo, and the WorldCare MRI technologist, Mr. Erik Peterson. Each observer independently identified the tumor margins on each axial image used for measurement of volume. Final volumes were then calculated. Data analysis was performed by the study statistician (figure 5). Since there is no "gold standard" among the 3 raters, inter-rater correlation coefficient (ICC) is used to summarize the agreement among the 3 raters. ICC is the proportion of variability explained by subject-to-subject variability. It ranges from -1 to 1 with 0 indicating only random concordance. With the 10 patients rated by all 3 raters, the ICC was 0.996.

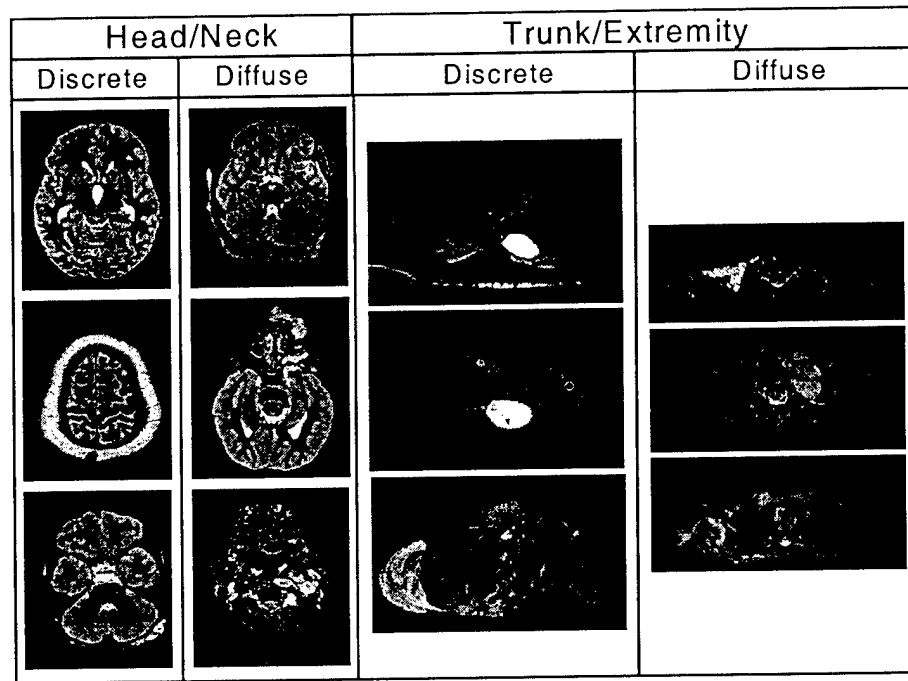


Figure 4. Images of tumors analyzed for reproducibility study. Six were head/neck tumors and six were from spine/extremities. Half the tumors in each group had discrete margins, and half had more diffuse margins.

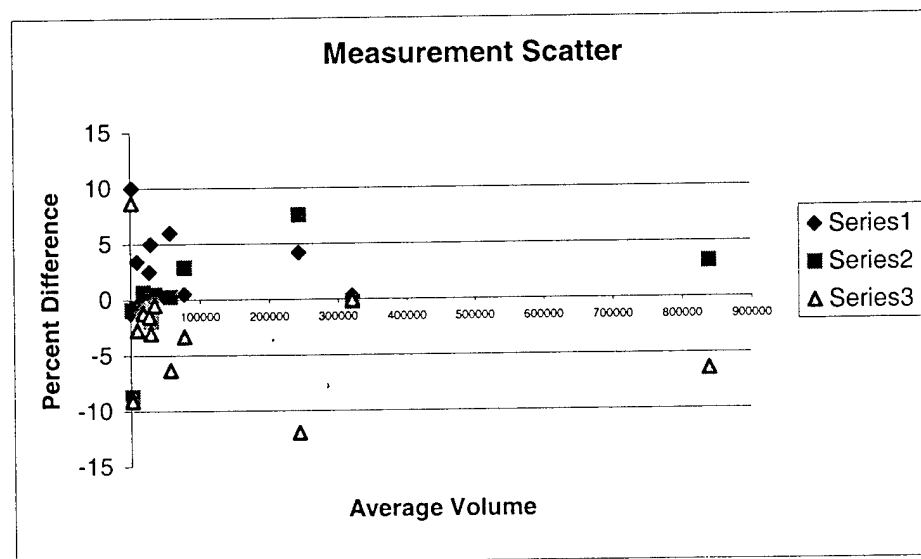


Figure 5. Scatter graph showing deviation of individual measurements of each of three observers from mean of the three. Series 1 and 2 are the two study radiologists and series 3 is the technologist. There was a tendency towards overestimation of volumes by radiologist 1 and underestimation by the technologist.

Task 3. Data Acquisition and analysis – Months 13-42

MRI's are sent from individual study sites in batches. The current status of MRI receipt is shown in the table below:

| University of British Columbia | Children's Hospital Boston | Klinikum Nord Ochsenzoll | St. Louis Children's Hospital | Children's Memorial Hospital | Children's Hospital of Oklahoma | Guys Hospital |
|--------------------------------|----------------------------|---------------------------|-------------------------------|------------------------------|---------------------------------|---------------|
| 100-0057-001 | 107-0021-500 | 160-0021-400 ² | 169-0001-001 | 177-0037-001 | 178-0001-001 | 187-0001-001 |
| 100-0061-001 | 107-0033-500 | 160-0083-400 ² | 169-0003-001 | 177-0142-001 | 178-0002-001 | 187-0002-001 |
| 100-0145-500 | 107-0050-500 | 160-0086-400 ³ | 169-0004-001 | 177-0207-001 | 178-0003-001 | 187-0003-001 |
| 100-0206-500 | 107-0053-500 | 160-0091-400 | 169-0006-001 | 177-0214-001 | 178-0003-002 | 187-0004-001 |
| 100-0207-500 | 107-0055-402 | 160-0094-400 | 169-0007-001 | 177-0224-001 | 178-0004-001 | 187-0005-001 |
| 100-0208-500 | 107-0123-500 | 160-0100-500 ² | 169-0008-001 | 177-0248-001 | 178-0005-001 | 187-0006-001 |
| 100-0210-500 | 107-0131-500 | 160-0124-400 ² | 169-0009-001 | 177-0298-001 | 178-0006-001 | 187-0007-001 |
| | 107-0160-500 | 160-0126-400 ² | 169-0010-001 | 177-0349-001 | | 187-0008-001 |
| | 107-0200-500 | 160-0146-500 | | 177-0367-001 | | 187-0009-001 |
| | 107-0316-500 | 160-0163-500 | | 177-0436-001 | | 187-0010-001 |
| | 107-0491-500 | 160-0243-400 | | 177-0455-001 | | 187-0012-001 |
| | 107-0520-001 | 160-0305-400 | | 177-0495-001 | | 187-0013-001 |
| | 107-0521-500 | 160-0341-400 ² | | | | 187-0014-001 |
| | 107-0524-500 | 160-0377-400 ² | | | | 187-0015-001 |
| | 107-0618-500 | 160-0404-400 | | | | 187-0016-001 |
| | | 160-0416-400 ² | | | | 187-0017-001 |
| | | 160-0425-400 | | | | 187-0018-001 |
| | | 160-0435-400 | | | | 187-0019-001 |
| | | 160-0436-400 | | | | 187-0020-001 |
| | | 160-0438-400 | | | | |
| | | 160-0439-400 ² | | | | |
| | | 160-0461-400 | | | | |
| | | 160-0470-500 | | | | |
| | | 160-0471-400 | | | | |

² = 2 scans

³ = 3 scans

Task 4. *Interpretation of Data – Months 43-48*

As a sample of volumetric data analysis, the image in figure 6 was analyzed by one of the project radiologists. The tumor is located in the peripheral nervous system (leg). The picture is followed by a table relevant tumor measurement analysis (see figure 7).

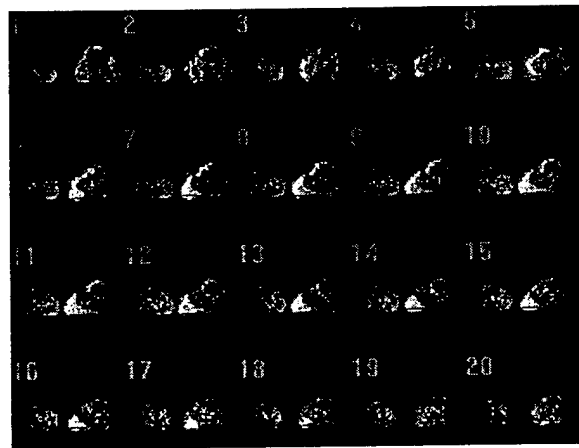


Figure 6. Sample images analyzed for tumor volume. The tumor areas are indicated by the red lines.

| Document | Mean | Max | Min | Std. Dev. | Area | Sum |
|--------------|------------|------|-----|------------|-------------|-------------|
| Slide 1 | 330.24295 | 528 | 54 | 84.933266 | 1751.098633 | 947467 |
| Slide 2 | 342.712036 | 539 | 87 | 73.911118 | 2066.650391 | 1160423 |
| Slide 3 | 353.597717 | 585 | 82 | 89.783112 | 2817.382813 | 1632207.125 |
| Slide 3 | 254.181824 | 409 | 138 | 74.17057 | 33.569336 | 13980 |
| Slide 4 | 319.796631 | 604 | 2 | 117.161003 | 2998.046875 | 1570841 |
| Slide 4 | 193.521744 | 319 | 83 | 65.442207 | 14.038086 | 4451 |
| Slide 4 | 270 | 359 | 171 | 52.364109 | 8.544922 | 3780 |
| Slide 5 | 292.94632 | 632 | 6 | 126.102142 | 5606.079102 | 2690712 |
| Slide 6 | 329.535828 | 686 | 3 | 123.60788 | 6857.299805 | 3702335 |
| Slide 7 | 382.763855 | 702 | 25 | 113.791824 | 6759.033203 | 4238727 |
| Slide 8 | 372.352936 | 659 | 28 | 117.654472 | 7299.804688 | 4453341 |
| Slide 9 | 401.170593 | 788 | 51 | 123.72673 | 7341.918945 | 4825681 |
| Slide 10 | 410.535065 | 732 | 53 | 118.107971 | 6759.643555 | 4546676 |
| Slide 11 | 442.351105 | 788 | 59 | 126.052376 | 6195.678711 | 4490306 |
| Slide 12 | 433.123291 | 779 | 51 | 129.369446 | 5974.731445 | 4239844 |
| Slide 13 | 485.977722 | 869 | 80 | 134.434494 | 5344.848633 | 4255707 |
| Slide 14 | 504.947113 | 912 | 59 | 132.067017 | 4558.71582 | 3771450 |
| Slide 15 | 575.947815 | 937 | 139 | 137.771805 | 3358.154297 | 3168865 |
| Slide 15 | 447.087555 | 653 | 209 | 86.783066 | 181.274414 | 132785 |
| Slide 15 | 408.20871 | 615 | 170 | 88.808098 | 336.303711 | 224923 |
| Slide 15 | 313.565216 | 465 | 169 | 84.806602 | 14.038086 | 7212 |
| Slide 16 | 559.766296 | 955 | 48 | 185.851852 | 3068.847656 | 2814505 |
| Slide 16 | 403.216217 | 596 | 177 | 79.879829 | 158.081055 | 104433 |
| Slide 17 | 506.058899 | 1026 | 30 | 208.278015 | 3180.541992 | 2637073 |
| Slide 18 | 476.825104 | 964 | 44 | 197.801544 | 2519.53125 | 1968334 |
| Slide 19 | 510.797852 | 1003 | 53 | 189.546677 | 1419.067383 | 1187605 |
| Slide 20 | 517.479675 | 1007 | 52 | 189.78772 | 1067.504883 | 905071.9375 |
| Document | Mean | Max | Min | Std. Dev. | Volume | Sum |
| Total Volume | 415.52 | 1026 | 2 | 153.02 | 876904 | 59698736 |

Figure 7. Relevant tumor measurement analysis is derived from the tumor borders of each slice. The first set of rows describes the transcribed tumor area of each slice (note: some slices may have multiple areas of interest). The last row describes the total three-dimensional tumor volume. To arrive at this number the total areas of each slice are multiplied by the slice thickness. Mean, Max, Min and Std. Dev. Refer to the pixel intensity. Area and Volume measurements are reported in millimeters.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of reproducibility study showing high inter-rater correlation coefficient, suggesting that volumetric analysis will provide reproducible data on tumor volumes.
- Substantial increase in study centers that have passed complete IRB approval (10) and steady increase in patient enrollment
- Addition of 9 new sites to study, more than compensating for dropout of 3 sites
- Consolidation of tissue bank and pathology review in single site with new protocol for submission of tissues available on study website
- Creation of study website at www.nfstudies.org
- Streamlining of IRB approval process to increase the speed of IRB approval of new sites
- Development of web-based system for obtaining updated information on status of MRI receipt and review

CONCLUSIONS

The major goals of this study have been to document the usefulness of volumetric MRI as a means of following the growth of plexiform neurofibromas, measuring the rate of growth of plexiform neurofibromas over time, and establishing a consortium of clinical centers to carry out clinical trials of treatment for plexiform neurofibromas in the future. Establishment of this consortium has been considerably more difficult than anticipated, due to the need to have every center approved both by a local IRB and the army IRB. Many minor and a few major differences in wording of informed consents have impeded many centers from completing the IRB approval process. We have tried to streamline this process, now, and expect that 19 centers will be approved by the first quarter of 2001. With this, however, we will have accomplished one of our major goals, to create a consortium of clinical centers, supported by a tissue repository, database, pathology review, MRI review, and statistical support. We have also taken a major step towards demonstration that the volumetric approach can be accomplished in spite of the complexities of imaging of plexiform neurofibromas by showing that three independent observers obtain similar volume measurements. The final goal, that of determining rate of neurofibroma growth, will take longer than expected due to the slow acquisition of IRB approval for all clinical centers, but this is expected to be accomplished as well, albeit after a longer period of study than initially anticipated. The first clinical trial of a farnesyl protein transferase inhibitor is about to begin under the auspices of the National Cancer Institute. We are involved in this study, using the same MRI protocol and tissue repository for that study. It is expected that additional clinical trials will begin over the next several years, and that the approaches and resources of this consortium will facilitate the efforts to test these drugs and determine treatment endpoints in NF1.

Appendix A

Plexiform Neurofibroma Tumor Repository

Introduction

The Washington University Tissue Procurement Facility will assist in the collection, storage, and processing of specimens from all study participants. This will include processing of whole blood specimens to aliquots of peripheral leukocyte cell pellets and serum, as well as collection and storage of fixed and fresh-frozen tissue specimens. Diagnostic blocks and fresh-fixed tissue specimens will be sent for centralized Neuropathology Review. Snap frozen tissue specimens will be stored for future research projects. All specimens will be stripped of patient identifiers upon entry into the facility (except for diagnostic blocks) and referenced in the facility database only by patient study number and a second, unique, specimen code number. The Washington University Department of Neurology (not the submitting institution) will be responsible for procurement costs (including shipping fees) on a per specimen basis and will also reimburse designated pathologists for procurement and submission of fresh tissue specimens.

Genomic DNA, RNA, protein lysates, and histological sections from collected research material will be made available to investigators after appropriate panel review. There will be a nominal specimen processing charge billed to each requesting investigator for samples distributed.

Below are the general collection protocols for each type of specimen that may be received by the Facility. Specific handling instructions will also be provided in each specimen procurement kit.

Instructions for Sample Collection - Blood Only

Purpose: To collect peripheral leukocytes and serum from study participants who are not otherwise scheduled for surgical removal of tumor tissue.

1. To obtain shipping materials and information for collecting blood specimens, please contact:

Tara Flynn
Research Study Coordinator
Partners Center for Human Genetics
Phone: (617) 525-5750
Fax: (617) 525-5757
Email: tflynn2@partners.org

2. If possible, please collect from each patient 5-10 cc of blood in a purple top (K-EDTA) tube and 5-10 cc of blood in a red top (clot or serum) tube. Label all tubes with the participant's study number **ONLY**. If the study number is not available, label the tubes with the participant's name and date of birth. **Store blood at 4 degrees until shipping. DO NOT FREEZE THE BLOOD.**
3. Please call the Specimen Procurement Facility at (314) 454-7615 prior to shipping. **Do not send specimens on Friday or the day preceding a holiday.** Instead, store blood samples at 4 °C until Monday or the day after the holiday. Ideally, blood drawing should be scheduled so that the specimens may be shipped immediately to the Specimen Procurement Facility.
4. Place labeled blood tubes in the Styrofoam mailer. Include the absorbent pad. Seal the mailer with waterproof tape and place the mailer inside the outer cardboard box. Place the entire box in the ziplock bag provided with the kit. Then, place the entire kit inside a FedEx specimen shipping bag. Attach the pre-printed FedEx label to the bag and arrange for pick-up using the institution's standard procedures. All shipments should be sent priority overnight.
5. Email Tara Flynn at tflynn2@partners.org to inform PCHG of sample collection.

Instructions for Sample Collection - Diagnostic Blocks Only

Purpose: To collect diagnostic, paraffin embedded specimen blocks from institutional pathology departments where fresh specimens are not available. These specimens will be used for central pathology review as well as a limited number of future research projects.

1. Call the Specimen Procurement Facility at (314) 454-7615 to obtain a FedEx billing number.
2. After complete evaluation and issuance of a final pathology report as per institutional protocol, all diagnostic paraffin blocks from enrolled study participants should be sent to the Specimen Procurement Facility.
3. Wrap each block, **individually**, in padded material such as bubble wrap. Include a copy of the final pathology report and indicate the NF study number (if known) on the report. Place all blocks and the report in an appropriately sized FedEx envelope. Attach a FedEx label to the envelope with the shipping address (see pg. I-5). Mark 'Bill to Recipient' and include the FedEx Billing number provided by the Specimen Procurement Facility. All shipments should be sent priority overnight. **Do not send blocks without appropriate padded material.**
4. Upon receipt, the Specimen Procurement Facility will store all diagnostic specimens in a 4°C inventory system with both the institution's original surgical pathology accession number and an additional, unique specimen code number.
5. Diagnostic blocks will be used for centralized Neuropathology review and approved research protocols. Specimens in diagnostic blocks will **not** be exhausted for research purposes. Upon written request, the Specimen Procurement Facility will return any diagnostic block to the submitting institution for clinical use within 24 hours of notification.
6. If submission of all diagnostic paraffin blocks is not possible or prohibited by participating Pathology departments, the submitting pathologist should request five (5) slides containing a 4 micron, unstained tissue section from each diagnostic block. Sections should be placed on positively-charged slides following standard institutional procedure. Each slide should be labeled with the institution's surgical pathology accession number, as it appears on the final pathology report. Slides should be packed in standard, plastic 5-slide mailers and wrapped individually in appropriate padded material. Slides and the final pathology report should be packaged as described above and mailed (via FedEx) to the Specimen Procurement Core. After centralized review, additional sections from select blocks may be requested from the institution by the Specimen Procurement Core / neuropathology review.

Instructions for Sample Collection - Fresh Frozen Tissue

Purpose: To collect snap frozen and fresh-fixed specimens for centralized Neuropathology review and future research investigations.

1. Three to five days prior to tumor resection, please call or fax the Specimen Procurement Facility. Provide the exact name and shipping address of the physician (probably a pathologist) responsible for tissue acquisition.

Phone: (314) 454-7615

Fax: (314) 454-5525

2. A specialized shipping module and shipping materials will be mailed via overnight express to the physician indicated. Detailed instructions for tissue procurement will be included in the kit.
3. To enable future molecular and biochemical analyses with the specimen, the participating institutional pathologist must receive the tumor tissue fresh, rather than "fixed in formalin". After resection, the tissue

should be transported from the O.R. to the pathologist within 30 minutes. The specimen must not be placed in formalin, but may be placed in normal saline, Ringer's solution, or any other physiologic buffer solution.

4. A representative and sufficiently large piece of the specimen should be fixed in formalin for paraffin processing as per the institution's standard policies and procedures. The pathologist should thoroughly sample the surgical specimen (at least one block per centimeter in greatest dimension) to his/her satisfaction. This material should be used to make the clinical diagnosis and, later, sent for central pathology review.
5. If tissue remains, an additional piece of tumor tissue 0.5-1 cm³ in size should be wrapped in aluminum foil and immersed in liquid nitrogen for 5 minutes. Tissue that is grossly necrotic, hemorrhagic, or cauterized should be avoided. If liquid nitrogen is not available, the specimen may be immersed in an isopentane cryobath available in most surgical pathology frozen section rooms. If using a cryobath, be certain that the temperature of the bath is at or below -40°C. As a last option, specimens may be frozen by complete immersion in an ethanol / dry-ice bath. Specimens should be left in the cryobath or dry ice bath for at least 15 minutes to ensure complete freezing. Specimens should **not** be frozen by placing fresh tissue in a -80°C freezer. Once frozen, foil- wrapped tissue should be placed in one of the ziplock bags provided. Be certain that the specimen bag is accurately and legibly labeled with the patient's study number and tissue site. If the patient's study number is not known, label the bag with the patient's first name, last name, and date of birth. Label the bag with a fine-point, black permanent marker. Once frozen, tissue may be stored in a -80°C mechanical freezer until shipping. This material will be shipped to the Specimen Procurement Facility on dry ice for future molecular and genetic research studies.
6. If tissue still remains, 2-4, 2 mm fragments, preferably from various sites representing a spectrum of gross appearances, should be placed in the provided glutaraldehyde specimen container. The container should then be sealed with parafilm.
7. If tissue still remains, another representative specimen (~ 1 cm³) should be placed in the provided formaldehyde specimen container. The container should then be sealed with parafilm. This specimen will be embedded at the Specimen Procurement Facility and used in the event that the submitting institution's specimen block is not available.
8. If tissue still remains, the remainder of the specimen should be divided into 1 cm³ segments and snap frozen as described in (5).
9. After performing the local institutional evaluation and issuing a pathology report, a copy of the pathology report, all patient specimens listed above (as applicable), and all diagnostic paraffin blocks should be packed in the dual-compartment shipping kit provided. If submission of all diagnostic paraffin blocks is not possible or prohibited by participating Pathology departments, the submitting pathologist should request five (5) slides containing a 4 micron, unstained tissue section from each diagnostic block. These should be prepared and packaged as discussed above.
10. Specimens may be sent to the Specimen Procurement Facility on Monday through Thursday for next day delivery. Frozen tissue specimens may be held at -80°C and fixed tissue and blood specimens may be held at 4°C until ready for shipment. For shipping frozen tissue, use approximately 4 lbs. of dry ice. Layer half of the dry ice on the bottom of one compartment of the dual compartment chamber, place frozen specimen bags on the dry ice, fill the compartment with the remaining dry ice, and replace the foam cover. Place the blood tubes in the Styrofoam blood container. Make certain to include the absorbent material in the container. Seal the container with sealing tape, place the container in the ziplock bag, and load the container in the second compartment of the shipping box. Place the formaldehyde and glutaraldehyde specimen containers, diagnostic blocks and/or unstained slides, a copy of the final pathology report, and the specimen bank submission form in this compartment as well.
11. Seal the shipping box with filament shipping tape. Complete the pre-printed Federal Express airbill, insert it into the plastic pouch, and attach the pouch to the top of the shipping box. Complete the dry ice label and stick this label and the biohazard label to the side of the box.

12. Arrange for Federal Express pick-up through your usual institutional procedure. Ship specimens to the address below.
13. On the day that specimens are sent to the specimen bank, please contact the bank by phone, fax, or e-mail to notify what is being sent and when the shipment is expected to arrive.

Ship All Specimens To: (this should match the address on pre-printed FedEx labels)

Tissue Procurement Core Facility
Washington Univ. School of Medicine
ATTN: Dr. Mark A. Watson
Kings Highway Bldg.; Room 2316
Barnes-Jewish Hospital North
216 South Kings Highway
St. Louis, MO 63110

For Questions Regarding Specimen Procurement, Contact:

Mark A. Watson M.D., Ph.D.
Assistant Professor of Pathology
Division of Laboratory Medicine / Box 8118
Washington University School of Medicine
660 S. Euclid Ave.
St. Louis, MO. 63110
Phone: (314) 454-7919
Fax: (314) 454-5525
E-Mail: watsonm@labmed.wustl.edu

Pathology Review

I. Procedures for shipping are located in Section I

II. Background

Our ability to design rational therapies for plexiform neurofibromas in NF1 is heavily dependent upon an improved understanding of the composition, biological properties, and growth characteristics of these tumors. The cellular constituency of plexiform neurofibromas is often complex and mixed, including Schwann cells, fibroblasts, perineurial and dendritic/monocytic cells. The patterns are further complicated due to the intrinsic intra-neural growth pattern as well as entrapment of native neural elements. This complex intermingling of cellular elements has impeded our most basic understanding of these tumors. It has also rendered molecular studies that require relatively pure cellular populations difficult to interpret. Therefore, combined morphologic, immunohistochemical, and molecular studies are needed to elucidate the histogenesis, growth patterns, and malignant evolution of these tumors.

The Neuropathology Review consists of two parts: the central diagnostic neuropathology review, and the accompanying light- and electron-microscopic effort to identify the actual cell populations comprising the tumor. All tissues received from surgical biopsies and/or resections reviewed by Dr. Arie Perry:

- (1) The neuropathology diagnosis reported from the submitting institution will be confirmed or amended as appropriate. This data along with the original pathology data from the submitting institution will be entered into the project's database. Since this 'centralized' pathology review process is not a part of patient management, data will not be issued to the submitting institution or pathologist.
- (2) Dr. Perry also will perform histochemical, immunohistochemical and electron-microscopic studies attempting to define the cell types, proliferation index, and extent of entrapped non-neoplastic elements comprising regions of plexiform neurofibroma.
- (3) In addition to the overall diagnosis, sampling adequacy will be determined for each frozen tumor sample used for subsequent research studies, in order to adequately interpret molecular/biochemical results obtained from these specimens.

With this combined approach, the range of cellular constituents and their neoplastic properties will be carefully documented in plexiform neurofibromas. Along with related assays being developed (see below), we will provide a better understanding of histogenesis, growth potential, and malignant transformation of these tumors, thus facilitating a rational approach for guiding patient management.

III. Approach

To accomplish these goals, the following strategy is proposed for submitting plexiform neurofibroma tissue:

A. Source of Samples: The primary source of tissue to be studied is expected to be from plexiform neurofibromas, plexiform nerve sheath tumors of indeterminate malignancy (e.g. cellular or atypical neoplasms falling short of the minimal criteria for MPNST), and malignant peripheral nerve sheath tumors (MPNSTs) submitted for biopsy from subjects enrolled in the natural history study. We expect 5-10 such samples per year. We will also try to obtain archival tissue blocks and samples of freshly biopsied tumors from patients who are not enrolled in the natural history study. This will provide a larger sample size of tumors for pathological study.

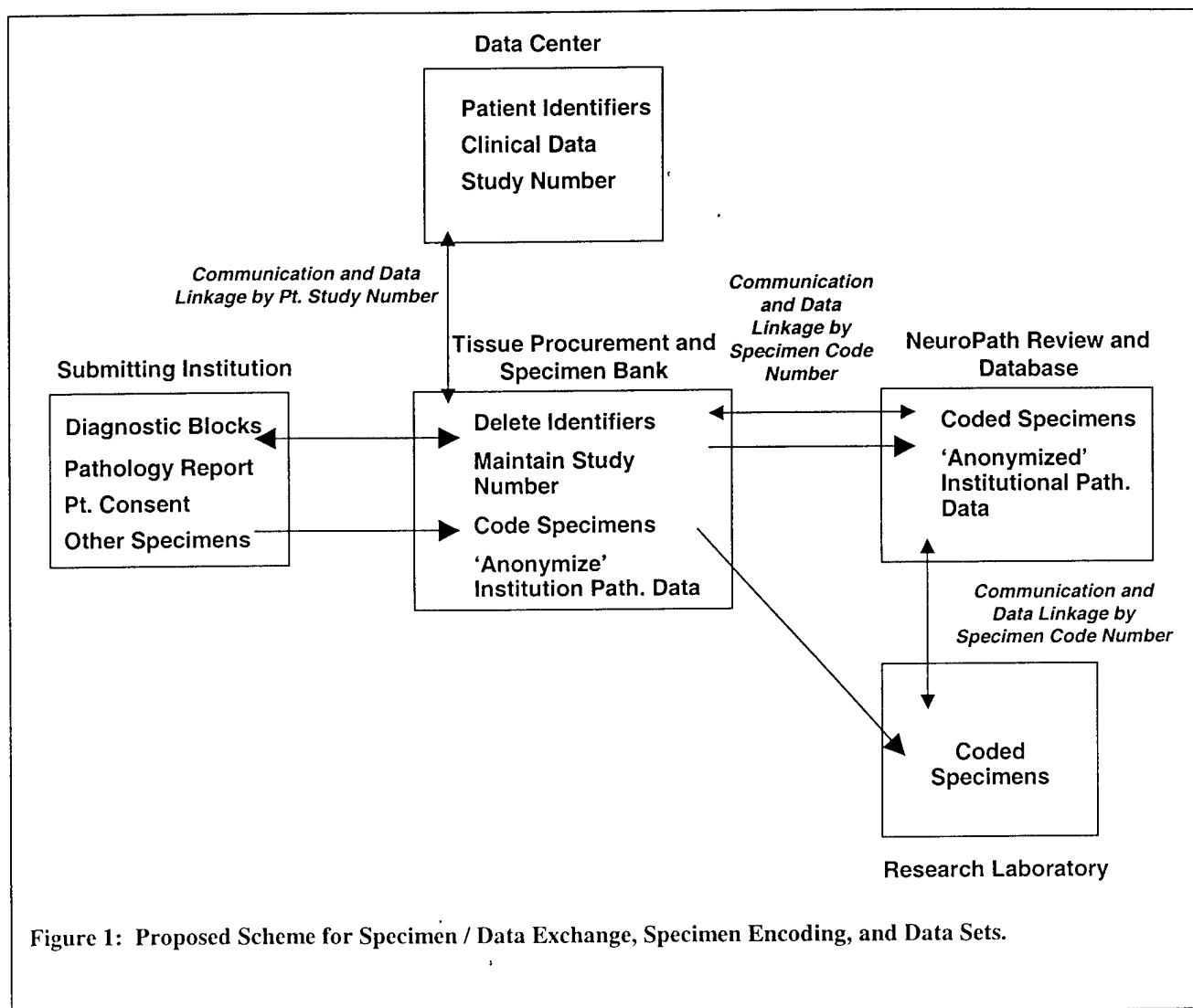
B. Tissue Acquisition: To assist in the collection of tissue specimens, the project's Tissue Procurement Facility will provide submitting pathologists with a complete specimen shipping kit. This kit will be sent to the submitting institution several days before the planned tissue resection. The kit will contain all materials and instructions for the proper collection and shipping of specimens to the Tissue Procurement Facility. Briefly, the steps for tissue collection are as follows:

1. To enable future molecular and biochemical analyses with the specimen, the participating institutional pathologist must receive the tumor tissue fresh, rather than "fixed in formalin". After resection, the tissue should be transported from the O.R. to the pathologist within 30 minutes. The specimen must not be placed in formalin, but may be placed in normal saline, Ringer's solution, or any other physiologic buffer solution.
2. A representative and sufficiently large piece of the specimen should be fixed in formalin for paraffin processing per the institution's standard policies and procedure. Pathologists will be instructed to thoroughly sample the surgical specimen (at least one block per centimeter in greatest dimension). This material will be used to make the clinical diagnosis and, later, sent for central pathology review.
3. An additional piece of tissue 0.5-1 cm³ in size will be snap frozen in liquid nitrogen or a -50°C histological bath. This material will be shipped to the Tissue Procurement Facility on dry ice for future molecular and genetic research studies.
4. If tissue remains, two to four 2-mm fragments, preferably from various sites representing a spectrum of gross appearances, will be placed in the provided glutaraldehyde container. These specimens will be used for electron microscopy studies.
5. If tissue still remains, another representative specimen will be placed in the provided formaldehyde container. This specimen will be embedded at the Tissue Procurement Facility and used in the event that the submitting institution's specimen block is not available.
6. If tissue still remains, the remainder of the specimen will be divided into 1 cm³ segments and snap frozen as described in (3).

After performing the local institutional evaluation and issuing a pathology report, a copy of the pathology report, a copy of the patient consent, and all patient specimens listed above (as applicable) will be packed in the dual-compartment shipping kit provided and sent by overnight express courier to Dr. Mark Watson at the Tissue Procurement Facility. All paraffin blocks should be sent with the other tissue specimens. If submission of all paraffin blocks is not possible or prohibited by participating pathology departments, H&E stained slides from each block will be sent instead. These slides will then be reviewed by Dr. Perry, who will then select 1 or 2 appropriate blocks to be sent for further study. Blocks will be returned to the submitting institutions upon completion of studies or within 24 hours of written request by the submitting institution.

Upon entry to the Tissue Procurement Facility, all specimens will be coded and recorded in the facility database. **Figure 1** diagrams the proposed flow of information and specimens, the coding scheme, and the residence of each dataset. This is a coded, double-broker model designed to maintain patient confidentiality while making meaningful research studies possible.

The Tissue Procurement Facility will forward appropriate coded specimens to Dr. Perry for centralized pathology review and other studies as described below. The remainder of the specimens (including paraffin blocks) will be stored by the Tissue Procurement Facility until needed for future research studies or recall by the submitting institution.



C. Pathological Studies: The paraffin blocks will be sectioned and the resulting slides will be stained with hematoxylin-and-eosin (overview). Selected blocks with the greatest degree of tumor purity and/or foci of malignant degeneration will be additionally stained with Masson's trichrome (collagen and myelin), alcian blue (acid proteoglycan), reticulin (basement membrane) and peroxidase-linked antibodies against neurofilament protein (axons), S-100 protein (Schwann cells), vimentin (mesenchymal elements including fibroblasts), chromogranin (entrapped or neoplastic ganglion cells), GFAP (some Schwann cells), Leu-7 (some Schwann cells), epithelial membrane antigen (perineurial cells), cytokeratin (epithelial differentiation), HMB-45 (melanin-containing cells), desmin (skeletal muscle differentiation), MIB-1 (Ki-67) antigen (growth fraction = proliferation index), collagen type IV (basement membrane), CD34 (endothelial cells and endoneurial dendritic/monocytic cells), muscle specific actin (some perineurial cells), CD68 (macrophages), p53 protein (overexpression due to mutation or protein stabilization common in MPNST components), and neurofibromin protein (antibody provided by consortium member, Dr. David Gutmann, Director of the Neurofibromatosis Clinic, Washington Univ. School of Medicine). Additional markers may be applied in select cases.

Glutaraldehyde-fixed tissue will be processed into epoxy resin for high-resolution light microscopy, and electron microscopy; ultrastructural criteria exist for the differential identification of Schwann cells, perineurial-like cells, and endoneurial fibroblasts and macrophages. All the attendant microscopy will be done, and the findings entered in the project's database, by Dr Perry.

D. Investigational Neuropathology Studies: Although initial studies will focus on routine morphologic, immunohistochemical, and ultrastructural characterization of submitted tumors, a number of additional novel studies are currently being developed. Recently, Drs. Perry and Gutmann have successfully applied a neurofibromin antibody to archival paraffin-embedded astrocytomas resected from patients with NF1. A similar approach will be utilized in our study of plexiform neurofibromas, enabling morphologic correlation and the determination of what proportion of cells have lost expression. This will be followed by the development of several dual-color immunohistochemical assays such as Neurofibromin/MIB-1, Neurofibromin/p53, S-100/MIB-1, S-100/Neurofibromin, etc. Results will determine for the first time, which cell types are actively proliferating, lack expression of neurofibromin, and/or overexpressing p53 protein. Dr. Perry has extensive experience with fluorescence *in situ* hybridization (FISH) studies in paraffin embedded tumor. Therefore, DNA probes are currently being developed against the *NF1* gene. Future FISH and potentially combined FISH/Immunohistochemistry assays will be utilized to identify specifically which cell types have deleted the *NF1* gene.

IV. Data Analysis

The Neuropathological Review Facility is intended to provide a resource for consistent pathological analysis of plexiform neurofibromas. This will, in the long run, facilitate better pathological classification of plexiform neurofibromas and permit correlations of pathological characteristics with clinical and cellular/molecular features. This is an important support service that will be needed for future clinical trials, given the lack of current information on the neuropathology of plexiform neurofibromas in the current medical literature. It is expected that the following questions will be addressed:

- What are the cell types present in plexiform neurofibromas?
- Are cell types different for plexiform neurofibromas obtained from different sites (e.g., cranial nerve, spinal nerve, peripheral nerve)?
- Does proliferation index correlate with growth or molecular/cellular characteristics?
- What is the range of neoplastic properties commonly seen in plexiform neurofibromas and how do these differ in benign and malignant lesions?
- Are there any immunohistochemical or DNA FISH markers (e.g. p53 protein expression or gene copy number) that may predict a high risk of subsequent malignant transformation in plexiform neurofibromas?